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(54) Title: METHODS AND COMPOSITIONS FOR ENHANCING DEVELOPMENTAL POTENTIAL OF OOCYTES AND ZYGOTES

(57) Abstract: The invention relates to compositions and methods for enhancing the developmental potential of oocytes or zygotes by increasing intracellular levels of replicative mitochondria in the oocytes or zygotes. In one aspect of the invention, the intracellular levels of replicative mitochondria are increased by introducing replicative mitochondria into the oocytes or zygotes. The oocytes may be fertilized to obtain a zygote with increased intracellular levels of replicative mitochondria. The methods and compositions may be used to improve in vitro fertilization and embryo transfer methods, and nuclear transfer techniques.



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**TITLE:** Methods and Compositions for Enhancing Developmental Potential of Oocytes and Zygotes

**FIELD OF THE INVENTION**

The invention relates to compositions and methods for enhancing the developmental potential of oocytes, zygotes, and preimplantation embryos.

**BACKGROUND OF THE INVENTION**

With *in vitro* fertilization (IVF) and other assisted reproductive technologies, about 50% of human embryos undergo a suicide program of active cell death and become fragmented. Some infertility patients produce only fragmented embryos, which appears to be the cause of their failure to conceive or carry a pregnancy to term.

In all mammals, including humans, zygote development and the first cleavage divisions depend upon maternal RNA and protein products accumulated during oogenesis. Reproductive failure can be attributed to the lack of cleavage in the developing embryo. This phenomenon can be traced to a defect in the composition of the oocyte cytoplasm. Maternal cytoplasmic components are involved in embryonic arrest, because the "2-cell block" in mice can be overcome by transplantation of ooplasm from zygotes of non-arresting strains into the zygotes of arresting strains (Muggleton-Harris *et al.*, Nature. 1982 Sep 30;299 (5882):460-2). Similar oocyte cytoplasm transfer experiments demonstrated improvement in the developmental capacity of immature eggs in mice (Flood *et al.*, 1990). Later, Cohen and colleagues obtained a pregnancy in a patient with a history of consistently fragmented embryos by transfer of donor oocyte cytoplasm into the patient's oocytes at the time of intracytoplasmic sperm injection (Cohen *et al.*, Lancet, 1997 Jul 19;350(9072):186-7). More recently, Lanzendorf *et al.* (Fertil Steril. 1999 Mar;71(3):575-7) demonstrated that frozen-thawed oocyte cytoplasm microinjected into oocytes, improved their developmental competency after fertilization, and resulted in a twin pregnancy in a patient who previously produced only fragmented embryos.

Nuclear transfer as a means of producing identical individuals (clones) has been successfully performed in several mammalian species including goat, sheep, pig, cattle, and mice. In all of these cases, efficiency of this technique is very low. While development of reconstituted embryos to the blastocyst stage is moderate (~40%, Ogura *et al.*, 2000 Biol. Reprod. 62(6):1579-84, 2000; Mol. Reprod. Development 57:55-59, 2000), live birth rate is unexpectedly low (1-7%, Ogura *et al.*, 2000, supra, Polejaeva *et al.* Nature 2000 Sep 7;407(6800):86-90). Moreover, extensive fetal and early neonatal death has previously been reported in offspring obtained by nuclear transfer (Rideout WM 3rd, Wakayama T, Wutz A, Eggan K *et al.* 2000 Nat Genet Feb;24(2):109-10).

Thus, there is a need to enhance the developmental potential of oocytes to improve reproductive technologies including nuclear transfer methods.

**SUMMARY**

The invention relates to a method for enhancing developmental potential of oocytes comprising increasing intracellular levels of replicative mitochondria in the oocytes. In an embodiment

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of the invention, the intracellular levels of replicative mitochondria are increased by introducing replicative mitochondria into the oocytes. A method of the invention may additionally comprise fertilizing the oocytes to obtain a zygote with increased intracellular levels of replicative mitochondria.

The invention also relates to a method for enhancing developmental potential of zygotes comprising increasing intracellular levels of replicative mitochondria in the zygotes. In an embodiment of the invention, the intracellular levels of replicative mitochondria are increased by introducing replicative mitochondria into zygotes.

The invention further relates to an oocyte or a zygote with increased intracellular levels of replicative mitochondria obtained from a method of the invention.

10 In a further aspect the invention relates to a composition comprising replicative mitochondria for enhancing developmental potential of oocytes or zygotes, and for treating and preventing heritable mitochondrial diseases. The composition may comprise cryopreserved mitochondria.

In another aspect, the invention provides a method for fertilizing oocytes comprising removing oocytes from a follicle of an ovary, introducing replicative mitochondria into the oocytes, and fertilizing the resulting oocytes with spermatozoa.

15 In a still further aspect the invention provides a method for storing and then enhancing the developmental potential of oocytes comprising cryopreserving immature oocytes, thawing the cryopreserved oocytes, and introducing replicative mitochondria into the oocytes. A method is also contemplated for enhancing the developmental potential of oocytes comprising cryopreserving replicative mitochondria, thawing the mitochondria, and introducing the replicative mitochondria into oocytes.

The methods and compositions of the invention improve the quality of the oocytes that are being fertilized and the quality of zygotes, to increase the rate of success in embryo development and ongoing pregnancy. The methods and compositions are particularly useful in enhancing the developmental potential of oocytes or zygotes with mitochondrial DNA mutations or abnormal mitochondrial metabolic activity.

25 In an aspect, the invention provides a method for improving embryo development after in vitro fertilization or embryo transfer in a female mammal comprising implanting into the female mammal an embryo derived from an oocyte or zygote containing increased intracellular levels of replicative mitochondria.

30 The invention also provides a method for reducing the detrimental effects of mitochondrial DNA mutations (e.g. deletion or missense mutations) in the progeny of an individual affected by such mutations comprising introducing into oocytes or zygotes from the individual replicative mitochondria that does not contain the DNA mutations (i.e. healthy mitochondria). The invention further provides an oocyte or a zygote comprising both mitochondria with mitochondrial DNA mutations, and purified and isolated replicative mitochondria that do not contain the mitochondrial DNA mutations (i.e. healthy mitochondria).

The invention also relates to a method for treating heritable mitochondrial diseases in the progeny of an individual affected by such diseases comprising introducing into oocytes or zygotes from the individual replicative mitochondria comprising mitochondria that does not contain the DNA mutations (i.e. healthy mitochondria).

5 In an aspect of the invention, the oocyte is a recipient oocyte in a nuclear transfer method. Thus, the invention relates to a method for enhancing developmental potential of recipient oocytes in a nuclear transfer method comprising introducing replicative mitochondria into the recipient oocytes. The invention also contemplates recipient oocytes comprising replicative mitochondria, and blastocysts, embryos, and non-human animals formed from the nuclear transfer methods of the invention. In  
10 conventional nuclear transfer methods, the donor nucleus is placed in an enucleated oocyte obtained from a different individual. Thus, mitochondria in the recipient oocyte have not-co-existed with the donor nucleus. Since mitochondria are always maternally inherited, their replication, transcription, translation, and function does not only depend on mitochondrial DNA, but is tightly intercalated with the nuclear genome that co-exists with the mitochondria. The invention by introducing replicative  
15 mitochondria into recipient oocytes enhances the developmental potential of the recipient oocytes. This is expected to increase the live birth rate in nuclear transfer methods.

In an embodiment, the invention provides a method of cloning a non-human mammalian embryo by nuclear transfer comprising

- 20 (a) introducing a donor cell nucleus derived from a donor cell of a non-human mammal, and replicative mitochondria preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the non-human mammal from which the donor cell nucleus is derived, into an enucleated recipient oocyte of the same species as the donor cell to form a nuclear transfer unit,
- (b) culturing the nuclear transfer unit to form an embryo.

25 The method may further comprise permitting the embryo to develop into a cloned mammal. Therefore, the invention also provides a method of cloning a non-human mammal by nuclear transfer comprising

- 30 (a) introducing a donor cell nucleus derived from a donor cell of a non-human mammal, and replicative mitochondria preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the non-human mammal from which the donor cell nucleus is derived, into a non-human mammalian enucleated recipient oocyte of the same species as the donor cell to form a nuclear transfer unit,
- (b) culturing the nuclear transfer unit to form an embryo;
- 35 (c) implanting the embryo into the uterus of a surrogate mother of said species, and
- (d) permitting the embryo to develop into the cloned mammal.

In yet another embodiment, a method of cloning a non-human mammalian fetus by nuclear

transfer is provided comprising the following steps:

- (a) introducing a donor cell nucleus from a donor cell of a non-human mammal, and replicative mitochondria preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the non-human mammal from which the donor cell nucleus is derived, into an enucleated recipient oocyte of the same species as the donor cell to form a nuclear transfer unit,
- (b) culturing the nuclear transfer unit until greater than the 2-cell developmental stage; and
- (c) transferring the cultured nuclear transfer unit to a host non-human mammal of the same species such that the nuclear transfer unit develops into a fetus.

The method may also comprise developing the fetus into an offspring.

In a further aspect the invention provides a recipient oocyte comprising a perivitelline space and a donor cell nucleus and replicative mitochondria preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the same individual from which the donor cell nucleus is derived, deposited in the perivitelline space.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a bar graph showing the effect of mitochondria injection on preimplantation embryo development.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The term "oocytes" refers to the gamete from the follicle of a female animal, whether vertebrate or invertebrate. Preferably, the animal is a mammal, and more preferably is a non-human primate, a bovine, equine, porcine, ovine, caprine, buffalo, guinea pig, hamster, rabbit, mice, rat, dog, cat, or a human. Suitable oocytes for use in the invention include immature oocytes, and mature oocytes from ovaries stimulated by administering to the oocyte donor, *in vitro* or *in vivo*, a fertility agent or fertility enhancing agent (e.g. inhibin, inhibin and activin, clomiphene citrate, human menopausal gonadotropins including FSH, or a mixture of FSH and LH, and/or human chorionic gonadotropins). In some embodiments of the invention, the oocytes are aged (e.g. from humans 40 years +, or from animals past their reproductive prime). The oocytes in some embodiments of the invention contain mitochondrial DNA mutations. Methods for isolating oocytes are known in the art.

In the nuclear transfer embodiments of the invention oocytes are used as recipient cells (such cells are referred to herein as "recipient oocytes"). The recipient oocytes are obtained from non-human mammals, in particular domestic, sports, zoo, and pet animals including but not limited to bovine,

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ovine, porcine, equine, caprine, buffalo, and guinea pigs, rabbits, mice, hamsters, rats, primates, etc.

The term "zygote" refers to a fertilized oocyte prior to the first cleavage division.

The expression "enhancing the developmental potential of oocytes" refers to increasing the quality of the oocyte so that it will be more capable of being fertilized and/or enhancing mitochondrial function or activity in the oocyte for subsequent development and reproduction. Increasing the quality of the oocyte, and thus the fertilized oocyte (e.g. zygote), preferably results in enhanced development of the oocyte into an embryo and its ability to be implanted and form a healthy pregnancy. The expression "enhancing the developmental potential of zygotes" refers to increasing the quality of the zygotes and/or enhancing mitochondrial function or activity in the zygotes for subsequent development and reproduction. Increasing the quality of the zygotes, preferably results in enhanced development of the zygotes into an embryo and their ability to be implanted and form a healthy pregnancy. Quality can be assessed by the appearance of the developing embryo by visual means and by the IVF or nuclear transfer success rate. Criteria to judge quality of the developing embryo by visual means include, for example, their shape, rate of cell division, fragmentation, appearance of cytoplasm, and other means recognized in the art of IVF and nuclear transfer.

"Spermatozoa" refers to male gametes that can be used to fertilize oocytes.

"Heritable mitochondrial diseases" refers to diseases caused by defects in mitochondrial DNA or by defects in nuclear genes that are important to mitochondrial function. Examples of mitochondrial diseases include but are not limited to Kearns-Sayre syndrome, MERRF syndrome (Myoclonic Epilepsy with Ragged Red Fibres), MELAS syndrome (Mitochondrial Encephalopathy, Myopathy, Lactic Acidosis and Stroke-like episodes), and Leber's disease (I. Nonaka, Current Opinion in Neurology and Neurosurgery, 5 (1992) 622).

The term "replicative microchondria" refers to a preparation of purified mitochondria that are capable of replicating during embryo development and increasing mitochondrial copy number or function. The replicative mitochondria is substantially free of other cytoplasmic components including nuclear DNA, mRNA, proteins, antioxidants, and organelles other than mitochondria. The replicative mitochondria preparations are at least 60% free, preferably 75% free, and most preferably 90% free from other cytoplasmic components. Preferably the replicative mitochondria preparations contain greater than 70%, more preferably greater than 80%, most preferably greater than 90% functional mitochondria. A replicative mitochondria preparation typically contains about 2,000 to 20,000 mitochondria in a volume of 5 to 15 pL.

For the non-nuclear-transfer embodiments of the invention, replicative mitochondria are preferably derived from any stem cell (e.g. hematopoietic, embryonic, trophoblastic, primordial germ cells) or from any immortalized cell line (e.g. cancer, or intentionally transformed somatic cells) of any species, preferably human. The cells are preferably free of the common mitochondrial deletion mutation found clinically in patients with KSS syndrome (i.e. deleted 4799bp region at nt 8470-13,447; see Simonnetti et al, 1992) and any other pathologic mitochondrial DNA mutation. Particular methods

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for preparing replicative mitochondria are illustrated herein and are described in Darley-Usmer VM., Rickwood D, Willson MT. Mitochondria, a Practical Approach, Oxford Washington DC., IRL Press, 1987, pp. 1-16.

5 Stem cells used to prepare the replicative mitochondria can be genetically modified by genetic engineering techniques. A transgene may be introduced into the cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, or microinjection. Suitable methods for transforming and transfecting cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks. (See also Nolta et al Blood. 1995 Jul 10 1;86(1):101-10; and Nolta et al Proc Natl Acad Sci U S A. 1996 Mar 19;93(6):2414-9; and Kohn et al Nat Med. 1998 Jul;4(7):775-80.). By way of example, a transgene may be introduced into cells using an appropriate expression vector including but not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses). Transfection is easily and efficiently obtained using standard methods including culturing the cells on a monolayer of virus-producing cells (Van der Putten, Proc Natl Acad Sci U S A. 1985 Sep;82(18):6148-52; Stewart et al. (1987) EMBO J. 6:383-388). Examples of genes that may be introduced into the stem cells include genes encoding cell death protectors such as Bcl-xL and McL-1.

15 Cryoprotective methods can be used to maintain maximum viability of the replicative mitochondria. Cryopreservation can be carried out in a medium containing for example dimethylsulphoxide, ethylene glycol, or glycerol or sucrose with 1,2-propanediol, or the mitochondria can be vitrified using cryoprotectants such as ethylene glycol and dimethyl sulphoxide. In an embodiment of the invention, the cryopreservation procedure involves cooling the mitochondria in a cryoprotective solution to an appropriate temperature (e.g. -176°).

25 Scanning and transmission electron microscopy can be used to assess the purity and morphology of a preparation. In addition the preparation can be analyzed for membrane mitochondrial potential and the total number and concentration of functional mitochondria present can be determined in accordance with conventional methods as described herein. Replicative ability of the mitochondria in a preparation can be determined using conventional techniques including restriction fragment polymorphism methods as described herein.

30 The present invention generally involves the use of replicative mitochondria to enhance the developmental potential of animal oocytes, especially mammals, including sports, zoo, pet, and farm animals, in particular dogs, cats, cattle, pigs, horses, goats, buffalo, rodents (e.g. mice, rats, guinea pigs), monkeys, sheep, and humans. In the nuclear transfer methods, replicative mitochondria are used to enhance the developmental potential of non-human recipient oocytes.

35 A method of the invention involves removing the oocytes from follicles in the ovary. This can be accomplished by conventional methods for example, using the natural cycle, during surgical intervention such as oophorohysterectomy, during hyperstimulation protocols in an IVF program, or

by necropsy. Oocyte removal and recovery can be suitably performed using transvaginal ultrasonically guided follicular aspiration.

After oocytes have been isolated, replicative mitochondria are introduced into the oocytes, or the oocytes can be cryopreserved for storage in a gamete or cell bank. If the oocytes are not  
5 cryopreserved the oocytes should be treated in accordance with the method of the invention preferably within 48 hours after aspiration. If the oocytes are frozen, they can be thawed when it is desired to use them and treated in accordance with a method of the invention.

Replicative mitochondria may be introduced into the oocytes (or zygotes) by conventional microinjection techniques or by other techniques such as electrofusion of mitochondria contained  
10 within liposomes or other suitable means.

After introduction, simultaneously with, or prior to the introduction of the replicative mitochondria, the oocytes are fertilized with suitable spermatozoa from the same species. The fertilization can be carried out by known techniques including sperm injection. Suitable human in vitro fertilization and embryo transfer procedures that can be used include in vitro fertilization (IVF)  
15 (Trounson et al. Med J Aust. 1993 Jun 21;158(12):853-7, Trounson and Leeton, in Edwards and Purdy, eds., Human Conception in Vitro, New York:Academic Press, 1982, Trounson, in Crosignani and Rubin eds., In Vitro Fertilization and Embryo Transfer, p. 315, New York: Academic Press, 1983); intracytoplasmic sperm injection (ICSI) (Casper et al., Fertil Steril. 1996 May;65(5):972-6); in vitro fertilization and embryo transfer (IVF-ET)(Quigly et al, Fert. Steril., 38: 678, 1982); gamete  
20 intrafallopian transfer (GIFT) (Molloy et al, Fertil. Steril. 47: 289, 1987); and pronuclear stage tubal transfer (PROST) (Yovich et al., Fertil. Steril. 45: 851, 1987).

The methods and compositions of the invention can be used to increase the success rate of embryo development. In particular, they can be used to reduce the detrimental effects of mitochondrial DNA mutations (e.g. deletion or missense mutations) or abnormal or deficient mitochondrial function  
25 in the progeny of an individual affected by such mutations or abnormal or deficient function, by introducing in oocytes or zygotes from the individual replicative mitochondria that comprises healthy mitochondria.

Mitochondrial DNA deletions or mutations usually result in impaired oxidative phosphorylation and clinical pathology related to muscle or neurologic tissues. For example Kearns-  
30 Sayre syndrome (KSS) or progressive external ophthalmoplegia is the result of a common 4799 bp deletion (Holt *et al.*, Ann Neurol. 1989 Dec;26(6):699-708) and Leber's hereditary optic neuropathy (LHON) is due to a missense mutation in the mtDNA (Wallace *et al.*, Science 242, 1427 (1998)). Therefore, injecting oocytes from individuals with these conditions with healthy replicative mitochondria, creating heteroplasmy, may prevent the detrimental effect of mtDNA missense or  
35 deletion mutations in the progeny.

The invention also contemplates improved nuclear transfer methods using replicative mitochondria. Nuclear transfer methods or nuclear transplantation methods are known in the literature



and are described in for example, Campbell et al, Theriogenology, 43:181 (1995); Collas et al, Mol. Report Dev., 38:264-267 (1994); Keefer et al, Biol. Reprod., 50:935-939 (1994); Sims et al, Proc. Natl. Acad. Sci., USA, 90:6143-6147 (1993); WO 94/26884; WO 94/24274, WO 90/03432, U.S. Pat. Nos. 4,944,384 and 5,057,420.

5           Methods for isolation of recipient oocytes suitable for nuclear transfer methods are well known in the art. Generally, the recipient oocytes are surgically removed from the ovaries or reproductive tract of a mammal, e.g., a bovine. Once the oocytes are isolated they are rinsed and stored in a preparation medium well known to those skilled in the art, for example buffered salt solutions

10           Recipient oocytes must generally be matured *in vitro* before they may be used as recipient cells for nuclear transfer. This process generally requires collecting immature (prophase I) oocytes from mammalian ovaries, and maturing the oocytes in a maturation medium prior to fertilization or enucleation until the oocyte attains the metaphase II stage. Metaphase II stage oocytes, which have been matured *in vivo*, may also be used in nuclear transfer techniques.

15           Enucleation of the recipient oocytes may be carried out by known methods, such as described in U.S. Pat. No. 4,994,384. For example, metaphase II oocytes may be placed in HECM, optionally containing cytochalasin B, for immediate enucleation, or they may be placed in a suitable medium, (e.g. an embryo culture medium), and then enucleated later, preferably not more than 24 hours later. Enucleation may be achieved microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm (McGrath and Solter, Science, 220:1300, 1983), or using functional enucleation  
20           (see U.S. 5,952,222). The recipient oocytes may be screened to identify those which have been successfully enucleated.

25           The recipient oocytes may be activated on, or after nuclear transfer using methods known to a person skilled in the art. Suitable methods include culturing at sub-physiological temperatures, applying known activation agents (e.g. penetration by sperm, electrical and chemical shock), increasing levels of divalent cations, or reducing phosphorylation of cellular proteins (see U.S. 5, 496,720).

30           A nucleus of a donor cell, preferably of the same species as the enucleated oocyte, is introduced into the enucleated recipient oocyte. The donor cell nucleus may be obtained from any mammalian cells. Donor cells may be differentiated mammalian cells derived from mesoderm, endoderm, or ectoderm. In particular, the donor cell nucleus may be obtained from epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, fibroblasts, and muscle cells. Suitable mammalian cells may be obtained from any cell or organ of the body. The mammalian cells may be obtained from different organs including skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organ, bladder, kidney and urethra.

35           The nucleus of the donor cell is preferably membrane-bounded. A donor cell nucleus may consist of an entire blastomere or it may consist of a karyoplast. A karyoplast is an aspirated cellular subset including a nucleus and a small amount of cytoplasm bounded by a plasma membrane. (See

Methods and Success of Nuclear Transplantation in Mammals, A. McLaren, Nature, Volume 109, June 21, 194 for methods for preparing karyoplasts).

Replicative mitochondria is introduced into the enucleated recipient oocyte. The replicative mitochondria is preferably derived from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, and most preferably from the same individual from which the donor cell nucleus is derived. Methods for preparing replicative mitochondria are described herein.

Donor cells may be propagated, genetically modified, and selected in vitro prior to extracting the nucleus, or the replicative mitochondria.

The nucleus of a donor cell and/or the replicative mitochondria may be introduced into an enucleated recipient oocyte using micromanipulation or micro-surgical techniques known in the art (see McGrath and Solter, supra). For example, the nucleus of a donor cell may be transferred to the enucleated recipient oocyte by depositing an aspirated blastomere or karyoplast under the zona pellucida so that its membrane abutts the plasma membrane of the recipient oocyte. This may be accomplished using a transfer pipette. Similar methods may be used to introduce the replicative mitochondria.

Fusion of the donor nucleus and the enucleated oocyte may be accomplished according to methods known in the art. For example, fusion may be aided or induced with viral agents, chemical agents, or electro-induced. Electrofusion involves providing a pulse of electricity sufficient to cause a transient breakdown of the plasma membrane. (See U.S. 4, 994,384). In some cases (e.g. with small donor nuclei) it may be preferable to inject the nucleus directly into the oocyte rather than using electroporation fusion. Such techniques are disclosed in Collas and Barnes, Mol. Reprod. Dev., 38:264-267 (1994).

The clones produced using the nuclear transfer methods as described herein may be cultured either in vivo (e.g. in sheep oviducts) or in vitro (e.g. in suitable culture medium) to the morula or blastula stage. The resulting embryos may then be transplanted into the uteri of a suitable animal at a suitable stage of estrus using methods known to those skilled in the art. A percentage of the transplants will initiate pregnancies in the surrogate animals. The offspring will be genetically identical where the donor cells are from a single embryo or a clone of the embryo.

The following non-limiting examples are illustrative of the present invention:

#### Example 1

Injection of a mitochondrial fraction obtained from a human myeloid cell line (HL-60) accelerated and/or facilitated preimplantation embryonic development. Murine zygotes were microinjected with either a mitochondrial fraction or a buffer at day 0.5 and further cultured in vitro until day 3.5. Embryos receiving mitochondria were twice as likely to form fully expanded or hatching blastocysts when compared with the buffer injected zygotes (45% versus 17%) (See Figure 1).

#### Example 2

**Assessment of mitochondrial function, mtDNA copy number and mtDNA deletion rates in human**

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**oocytes of various ages and in human embryos showing preimplantation developmental defects.**

Patients with a history of either delayed embryo development (6-cell stage or less at 72 hours post insemination) or persistent embryo fragmentation resulting in only Grade 4 or 5 embryos (presence of cellular fragments filling at least 30% of total embryo volume) will be included in the study. At the time of retrieval, approximately 20% of oocytes are immature and thus unsuitable for fertilization using ICSI. These oocytes will be used in order to determine whether these patients have a maternal predisposition towards abnormal embryonic development that can be attributed to mitochondria. Rates of mitochondria dysfunction will be compared to immature oocytes obtained from patients with known history of normal embryo development. In addition, fragmented embryos, unsuitable for transfer, will be analyzed and their mitochondrial status compared between these two groups. The effect of maternal age will also be determined by examining mitochondrial normality in patients aged 25-30 years, 30-35 years and 35 above. The following experiments are proposed.

*A/ Mitochondrial function:* Changes in mitochondrial membrane potential reflect mitochondrial function since energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane and is used to drive ATP production. Disruption of mitochondrial membrane potential is one of the first signs of apoptosis in many somatic cells. Briefly, oocytes and embryos will be incubated with a fluorochrome (DePsipher, R&D Systems) that allows simultaneous detection of mitochondria with disrupted (non-functional) and maintained mitochondrial potential. Samples will be analyzed using a deconvolution microscope and the amount of fluorescence will be recorded using Delta Vision software package (Silicon Graphics). In dying cells or those with disrupted membrane potential, the dye will remain in its monomeric form in the cytoplasm and the mitochondria will appear green, whereas in healthy cells the dye aggregates in the mitochondria will appear red. Furthermore, this technique can be used to estimate mitochondrial copy number based on the total amount of fluorescence emitted on both channels. The immature (GV and MI stage) oocytes obtained from the ICSI program, unfertilized oocytes from IVF, and spare embryos donated to research will be analyzed.

*B/ Mitochondrial copy number:* In order to determine whether recurrent embryo fragmentation observed in some patients could be attributed to insufficient mitochondrial copy number within maternal stores, semi-quantitative PCR (Chen *et al.* 1995 Am J Hum Genet 57, 239-47) will be used to estimate approximate mtDNA copy number. After staining and assessment of mitochondrial function, individual oocytes or embryos will be placed in 20 µl of PBS and stored in -70°C. Before PCR, samples will be boiled and 1/10 of the volume of the lysate will be used as a template for the PCR reaction.

*C/ mtDNA deletions:* Although the above studies will determine the viability and abundance of the mitochondria, a further assessment can be done using PCR to semi-quantitatively assess mtDNA deletions in the same population of human oocytes and embryos used above. Different PCR primer sets, encompassing all regions of the mitochondrial chromosome, have been designed and the

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proportion of mitochondria with a deletion in any part of the chromosome will be determined using the approach of Zhang *et al.* (Biochem Biophys Res Commun 1996 Jun 14;223(2):450-5). This method of scanning the whole chromosome with multiple primer sets will circumvent the problems previously observed with very long mtDNA PCR (Kajander *et al.*, Biochem Biophys Res Commun 1999 Jan 19;254(2):507-14). Preliminary results have shown that the 4799 bp common deletion can be easily identified. In addition, amplified products will be subcloned and sequenced in order to identify specific deletions that could be associated with activation of PCD.

Expected Outcome. Information about mitochondrial function, mtDNA status and an estimate of mtDNA copy number will be obtained. This will allow comparison of different oocytes and embryos in order to determine whether there might be a predisposition towards mitochondrial dysfunction in some infertile patients. This data will also be analyzed with respect to increased maternal age and confirm previous reports of a higher rate of mtDNA mutations associated with reproductive senescence.

### Example 3

#### 15 Isolation of mitochondria and mouse models of embryo demise

The ability of an enriched fraction of mitochondria, isolated from both somatic cells and different types of stem cells, to enhance developmental potential and to suppress apoptosis following injection into oocytes will be assessed. The cells used for these experiments will include murine embryonic stem (ES) cells, murine and human trophoblastic stem (TS) cells, and human or murine CD34+/CD38- hematopoietic stem cells and granulosa cells. ES and TS cells will be grown *in vitro* under standard culture conditions (Hadjantonakis *et al.* Mech Dev. 1998 Aug;76(1-2):79-90, Tanaka *et al.* Science. 1998 Dec 11;282(5396):2072-5). The nucleated cells obtained from human umbilical cord blood of healthy donors will be isolated using a Ficoll gradient. CD34+/CD38- cells will be separated using a cell depletion magnetic column. Equivalent (but adult rather than fetal) cells can also be obtained from murine bone marrow of adult animals (Ploemacher *et al.* Exp Hematol. 1989 Mar;17(3):263-6). The somatic cell source will be luteinized granulosa/cumulus cells isolated from follicular fluid during oocyte retrieval for IVF or from ovaries of hormonally primed mice (Trbovich *et al.* Cell Death Differ. 1998 Jan;5(1):38-49). An enriched mitochondrial fraction can be isolated from all stem cell types and from granulosa cells using the method of Rickwood (Darley-Usmer VM., Rickwood D, Willson MT. Mitochondria, a Practical Approach, Oxford Washington DC., IRL Press, 1987, pp. 1-16). Briefly, cells are suspended in a sucrose-based buffer and lysed using a glass homogenizer. The nuclei are pelleted and the mitochondrial fraction is further enriched and purified using a continuous Percoll gradient to separate damaged from intact mitochondria and to eliminate most cellular debris. Scanning and transmission electron microscopy will be used to assess the purity and morphology of the mitochondrial fraction. The maintenance of membrane mitochondrial potential will be analyzed by DePsipher dye as described above in Example 1, coupled with FACS analysis for rapid calculation of the total number and concentration of both functional and damaged mitochondria

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present. Only fractions containing greater than 90% functional mitochondria will be used in the subsequent studies.

a) Ability of mitochondria to suppress fragmentation in FVB strain mouse oocytes cultured in vitro.

5 Mature oocytes of FVB strain mice undergo a very high rate (~75%) of spontaneous  
fragmentation within 48 hours when cultured in vitro (Morita *et al.* Dev Biol. 1999 Sep 1;213(1):1-17).  
This model will be used to test each mitochondria enriched fraction for its ability to suppress oocyte  
fragmentation. Ovulated oocytes will be stripped of their cumulus cells and will be injected with  
mitochondria enriched fraction in a dose response fashion according to the technique of Van Blerkom  
*et al.* (Hum Reprod. 1998 Oct;13(10):2857-68). It has been estimated that mature oocytes contain  
10 about 100,000 mitochondria (Jansen and de Boer, Mol Cell Endocrinol. 1998 Oct 25;145(1-2):81-8).  
Between 2000 and 20,000 mitochondria in a volume of 5 to 15 picoL will be injected. A control group  
of oocytes will be left intact or injected with either buffer used for suspension of mitochondria, or with  
the mitochondria depleted fraction. Damaged mitochondria obtained from the percoll gradient will also  
be injected to determine possible negative effects of damaged mitochondria on oocyte survival. All  
15 oocytes will then be cultured and scored for fragmentation at 24 and 48 hours. This model will be used  
to confirm the optimal number and type of mitochondria to inject to protect against fragmentation  
Expected Outcome: It is expected that mitochondria derived from stem cells will be successful in  
preventing fragmentation, and will have the benefit of potential replicative ability.

b) Does injection of mitochondria from stem cells into normal mouse zygotes fertilized in vitro provide  
20 long-lasting protection from cell death?

Increased maternal age and fertilization in vitro combines to result in an apoptosis rate of  
about 30% in murine zygotes, and to a higher cell death index at the blastocyst stage, compared to  
zygotes obtained from young mothers fertilized in vivo (about a 2% fragmentation rate) (Jurisicova  
*et al.* Mol Hum Reprod. 1998 Feb;4(2):139-45). Moreover, analysis of cell death rates in human  
25 blastocysts demonstrated that approximately 30% of embryos preferentially eliminated the inner cell  
mass or activated cell death in the majority of cells. To assess if injection of mitochondria can prevent  
apoptosis in zygotes and also provide protection during the later developmental stages, zygotes from  
aged mice (ICR strain 44 weeks old) will be injected with an enriched fraction of mitochondria and  
their development to the blastocyst stage will be observed in vitro. The number of mitochondria to be  
30 injected will be estimated using the methods set out in the previous experiment, and the concentration  
will be fine tuned if necessary. At day 4.5, blastocyst cell numbers and cell death rates will be recorded,  
with particular attention to the inner cell mass.

Further studies will examine the impact of mitochondrial injection on protection from cell  
death caused by various toxicants as an artificial trigger of apoptosis. In particular, whether  
35 mitochondrial injection can prevent apoptosis induced by treatment with doxorubicin (Bergeron *et al.*  
1998 Gen. Dev. 12, 1304-1314), hyperglycaemia (Moley *et al.* Nat Med 1998 Dec;4(12):1421-4) and  
DMBA, which have all been shown to activate the cell death pathway during blastocyst formation, will

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be investigated. In these experiments, zygotes injected with appropriate mitochondria will be cultured in KSOM medium until they reach the early blastocyst stage, when the experimental treatment will be performed in vitro with either doxorubicin (200nM), glucose (30mM) enriched medium or with DMBA (1µM). Zygotes injected with buffer or with mitochondria-depleted fractions that develop to the blastocyst stage will be used as controls. At 24 hours the toxicant addition, blastocyst cell number and cell death index will be determined as previously described (Jurisicova *et al.* 1998, *supra*).

Expected outcome. Somatic cell mitochondria have been shown to be diluted out by subsequent cell divisions of preimplantation embryos, and are non-detectable by the blastocyst stage (Ebert *et al.* 1989, *J Reprod Fertil. Jan;82(1):145-9* 9). Stem cell mitochondria should behave more like oocyte mitochondria, which have been demonstrated by Van Blerkom *et al.* (Hum Reprod. 1998 Oct;13(10):2857-68) to be detectable at least 80 hours after injection into mouse oocytes. If the donor stem-cell mitochondria are replicative and persist to the blastocyst stage, protection from spontaneous apoptosis in vitro, and decreased rates of cell death following toxicant administration should be observed.

c) Assessment of normal development of mice derived from zygotes injected with stem-cell mitochondria.

To determine if mitochondria injection may compromise normal development and life span, FVB zygotes will be injected with various stem or somatic cell mitochondria-enriched fractions as described above and transferred into pseudopregnant females. At least 20 progeny in each group will be obtained. The offspring will be followed over an 18-month period for detection of any developmental abnormalities, reproductive dysfunction, or reduced life span, that might be attributable to a deleterious effect of donor mitochondria injection on pre and postnatal development. Moreover, since 75% of oocytes from this strain normally undergo apoptosis in vitro, female offspring will also be assessed for their oocyte fragmentation rate in vitro to determine if the donor mitochondria have replicated in the offspring, producing heteroplasmy. All the parameters will be compared with offspring generated from sham injected zygotes.

Another way to determine the replicative ability of donor stem-cell mitochondria is to utilize restriction fragment length polymorphism (RFLP) in mtDNA, as has been reported between strains C57Bl6/J and NZB/BINJ (Jackson laboratories) (Meirelles and Smith, Genetics 1998 Feb;148(2):877-83). The FVB strain will be examined to determine if it contains mtDNA RFLP similar to either of the two strains and based on these results, TS or ES cell lines will be derived from the opposite strain. Mitochondria enriched fraction from these genetically distinct cells will be injected into FVB zygotes. The replicative potential of injected mitochondria can then be confirmed in the offspring by determining the RFLP status of the isolated mitochondria.

Expected outcome. The offspring created by donor stem-cell mitochondrial injection should be phenotypically normal, with normal lifespan. These mice may have improved reproductive function, and decreased oocyte apoptosis in vitro, if the donor mitochondria are replicative and capable of

creating heteroplasmy. The ability to create heteroplasmy is critical to the success of any future clinical studies aimed at correcting heritable mitochondrial diseases.

D) No rescue of embryo fragmentation mediated by DNA damage.

A subset of both male and female gametes contain damaged DNA (Sun *et al.*, Biol Reprod. 1997 Mar;56(3):602-7, Lopes *et al.*, Fertil Steril 1998 Mar;69(3):528-32). Results of Twigg *et al.* (Hum Reprod 1998 Jul;13(7):1864-71) with ROS-induced sperm DNA damage clearly demonstrated the ability of such sperm to undergo decondensation and pronuclear formation, suggesting that early stages of embryo development may occur even if the paternal DNA is fragmented. It is not desirable to rescue embryos with chromosomal abnormalities. Genetic analysis of the cell death pathway in murine germ cells, suggests that one can prevent apoptosis in the female germ line if the trigger is lack of survival signals, but not if the initiating factor is DNA damage. A model developed by Doerksen and Trasler (Biol Reprod 1996 Nov;55(5):1155-62) will be used in which male mice are treated with 5-azacytidine (5-AZC), a drug that interferes with DNA methylation and induces sperm DNA damage. Female mice, when mated to these treated males, produce embryos with a high rate of fragmentation and low pregnancy rates secondary to chromosomal damage (Doerksen and Trasler, 1996, supra). In this experiment, male animals will be treated with 5-AZC (4 mg/kg for 3 weeks), sperm will be collected from the cauda epididymus and injected together with stem cell mitochondria or buffer into the oocytes of FVB strain mice.

Expected outcome. Failure of mitochondrial injection to protect against embryo fragmentation in this model will confirm that human embryos will not be rescued in which the cell death pathway has been activated by DNA damage. In addition, the report of injection of donor oocyte cytoplasmic into the oocytes of 7 patients by Cohen and his colleagues (Lancet 1997 Jul 19;350(9072):186-7) described 2 couples in which no improvement in embryo quality was seen. These 2 couples were the only ones in which the men had severe oligoasthenospermia, which has been shown to be associated with a high degree of sperm DNA damage (Sun *et al.*, Biol Reprod 1997 Mar;56(3):602-7; Lopes *et al.*, Fertil Steril 1998 Mar;69(3):528-32, Hum Reprod 1998 Mar;13(3):703-8). The presence of DNA fragmentation in the sperm may explain why the injections were unsuccessful in these two cases.

**Example 4**

**Overexpression of Mcl-1 and Bcl-xL in stem cell mitochondria to enhance suppression of cell death in mouse and human embryos.**

In mouse and human oocytes and embryos, two cell death protectors, Bcl-xL and Mcl-1, both of which localize to mitochondria, are abundantly expressed. Variable levels of maternally stored transcripts have been observed for these two proteins in human oocytes suggesting that variation in these proteins may lead to varying susceptibility to cell death triggers.

Transfected ES cells that overexpress Bcl-x<sub>L</sub> or Mcl-1, driven by a ubiquitous chicken b-actin promoter (pCAX - Hadjantonakis *et al.*, 1998, supra) will be created. Transfected lines will be selected based on their resistance to neomycin and will be assessed for protein levels of Mcl-1 or Bcl-x<sub>L</sub> within

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their mitochondrial fraction using western blot analysis. Cytochrome C, another mitochondrial-localized protein, will be used as a loading control in order to show enhanced levels of Bcl-xL and Mcl-1 in mitochondria enriched fractions. Upon establishing increased levels of protein expression on the mitochondrial membranes within these cells, mitochondria will be isolated and used in similar experiments to those described above. Therefore, early embryos can be augmented with more functional mitochondria, but also with mitochondria containing a higher protein content of either Bcl-x<sub>L</sub> or Mcl-1.

Expected Outcome. If these transfected mitochondria are superior in suppressing cell death compared to their non-transfected counterparts, the importance of either Bcl-x<sub>L</sub> or Mcl-1 in the prevention of apoptosis and normal embryo development in this model will be established.

#### Example 5

##### Injection of mitochondria into human oocytes at the time of ICSI and rescue of fragmented embryos.

Twenty patients who have undergone two cycles of IVF and who produce only very fragmented embryos (Grade 4 or 5) or embryos with delayed development (6 cells or less at 72 hours post fertilization), will be recruited for a pilot study. Women must have normal day 3 serum FSH concentrations (<10 IU/L in our lab) initially, but if the results of preliminary studies appear promising, older women with elevated serum FSH concentrations will be enrolled for the procedure as well. Ovulation induction will consist of a long GnRH-agonist protocol with various human menopausal gonadotropins as previously described (Greenblatt *et al.*, Fertil Steril. 1995 Sep;64(3):557-63). Cycles will be monitored using a combination of transvaginal ultrasound and serum estradiol measurements. Human chorionic gonadotropin will be administered at 36 h before oocyte retrieval. Oocytes will be collected transvaginally under ultrasound guidance. Following oocyte retrieval, the cumulus cells will be removed by exposing the cumulus corona-oocyte complex to hyaluronidase in modified HTF medium. Each oocyte will be assessed for maturity and those with a first polar body present (MII) selected for ICSI. Immature oocytes will be used for determination of mitochondrial function and mtDNA copy number and mutations as described in Example 2. Spermatozoa will be prepared on the day of oocyte retrieval as previously described (Sun *et al.*, Biol Reprod. 1997 Mar;56(3):602-7). The ICSI procedure to be used in this study has been previously described in detail (Casper *et al.*, 1996, supra). All microinjection procedures will be carried out on the heated stage of an inverted microscope (magnification x200 or x400). For the microinjections, a morphologically normal, motile sperm will be selected from a sperm/PVP droplet and immobilized. Oocytes from each patient will be divided into two groups. Oocytes in group one will be injected with a single sperm as previously described (Casper *et al.*, 1996, supra). Oocytes in group 2 will be injected with a single sperm aspirated into the injection pipette together with between 5,000 and 20,000 intact mitochondria from human umbilical cord blood-derived hematopoietic stem/progenitor cells prepared as described above. The volume for injection including both sperm and mitochondria will be kept to a maximum of 15 picoL. Following injection,



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oocytes will be transferred into a 100 µl droplet of HTF medium supplemented with 5% human serum albumin in a plastic 60 x 15 mm petri dish, covered with mineral oil and incubated in a humidified 5% CO<sub>2</sub> environment at 37°C. Cultured oocytes will be assessed for the presence of two pronuclei, indicative of normal fertilization at 16-18 h after ICSI. Embryo development and grading according to the method of Veeck (1991; Acta Eur Fertil. 1992 Nov-Dec;23(6):275-88) will be performed daily. The embryo score (cell number X 1/grade) will be determined for each embryo at 48, and 72 hours, and cell number estimated at 96 and 120 hours. Morphologically normal appearing expanded blastocysts will be transferred at day 5 post-fertilization. If normal embryo development occurs in any of the control injected oocytes, they will be transferred first. The pregnancies obtained by this technique will be followed closely and the patients advised to consider amniocentesis to rule out a gross chromosomal abnormality. Babies born as a result of this procedure will have their cord blood collected and stored for determination of mitochondrial heteroplasmy if possible (ie. if a mtDNA mutation is detected in the unfertilized oocytes), and which may be responsible for the embryo fragmentation or delayed development seen initially in these patients. The babies will also be followed with assessment for normal development at birth, and at intervals thereafter for as long as the parents agree.

Expected outcome. Group 1 oocytes should result in embryos with delayed development or which are completely fragmented, consistent with the patient's past history. In group 2 oocytes, injection of an enriched fraction of stem cell mitochondria will allow normal development to the blastocyst stage with intrauterine transfer and pregnancy in some patients.

The present invention is not to be limited in scope by the specific embodiments described herein, since such embodiments are intended as but single illustrations of one aspect of the invention and any functionally equivalent embodiments are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety. All publications, patents and patent applications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the methodologies etc. which are reported therein which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a gene" includes a plurality of such genes.

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**WE CLAIM:**

1. A method for enhancing developmental potential of oocytes or zygotes comprising increasing intracellular levels of replicative mitochondria in oocytes or zygotes.
- 5 2. A method as claimed in claim 1 wherein the intracellular levels of replicative mitochondria are increased by introducing replicative mitochondria derived from stem cells or an immortalized cell line.
3. A method as claimed in claim 2 wherein the replicative mitochondria are introduced by microinjection or electrofusion.
- 10 4. A method as claimed in claim 2 or 3 wherein the stem cells have been genetically modified.
5. A method as claimed in claim 1, 2 or 3 wherein the replicative mitochondria comprise mitochondrial DNA free of deletions or mutations.
6. A method as claimed in any of the preceding claims wherein the replicative mitochondria are at least 60% free, preferably 75% free, and most preferably 90% free from other cytoplasmic components.
- 15 7. A method as claimed in claim 2 wherein the replicative mitochondria derived from the stem cells or immortalized cell line contains about 2,000 to 20,000 mitochondria.
8. A method as claimed in any of the preceding claims wherein the oocytes or zygotes are from sports, zoo, pet and farm animals.
- 20 9. A method as claimed in any of the preceding claims wherein the developmental potential of human oocytes are enhanced.
10. An oocyte or zygote with increased intracellular levels of mitochondria obtained from a method as claimed in any of the preceding claims.
11. A method as claimed in claim 9 further comprising fertilizing the oocytes to obtain a zygote with increased intracellular levels of replicative mitochondria.
- 25 12. A zygote with increased intracellular levels of mitochondria obtained from a method as claimed in claim 11.
13. A composition comprising replicative mitochondria for enhancing developmental potential of oocytes and zygotes.
- 30 14. A composition as claimed in claim 13 wherein the replicative mitochondria is derived from stem cells or an immortalized cell line.
15. A composition as claimed in claim 13 wherein the replicative mitochondria is derived from differentiated mammalian cells.
16. A method for reducing the detrimental effects of mitochondrial DNA mutations in the progeny of an individual affected by such mutations comprising introducing into oocytes or zygotes from the individual replicative mitochondria comprising healthy mitochondria.
- 35 17. A method as claimed in claim 16 wherein the replicative mitochondria comprise mitochondrial

DNA free of deletions or mutations resulting in impaired oxidative phosphorylation and clinical pathology related to muscle or neurologic tissues.

18. A method for improving embryo development after in vitro fertilization or embryo transfer in a female mammal comprising implanting into the female mammal an embryo derived from an oocyte or zygote containing increased intracellular levels of replicative mitochondria.
19. A method of cloning a non-human mammal by nuclear transfer comprising
  - (a) introducing a donor cell nucleus derived from donor cell of a non-human mammal, and replicative mitochondria preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the same non-human mammal from which the donor cell nucleus is derived, into an enucleated recipient oocyte of the same species as the donor cell to form a nuclear transfer unit,
  - (b) culturing the nuclear transfer unit to provide an embryo;
  - (c) implanting the embryo into the uterus of a surrogate mother of said species, and
  - (d) permitting the embryo to develop into the cloned mammal.
20. A method to produce viable embryos of a non-human mammal comprising:
  - (a) introducing a donor cell nucleus derived from a donor cell of a non-human mammal, and replicative preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the same non-human mammal from which the donor cell nucleus is derived, into an enucleated recipient oocyte of the same species as the donor cell to form a nuclear transfer unit,
  - (b) culturing the nuclear transfer unit to provide an embryo.
21. A method of cloning a fetus of a non-human mammal by nuclear transfer comprising the following steps:
  - (a) introducing a donor cell nucleus derived from a donor cell of a non-human mammal, and replicative preferably from the same species as the donor cell, more preferably from the same species and cell type as the donor cell, most preferably from the same non-human mammal from which the donor cell nucleus is derived, into an enucleated recipient oocyte of the same species as the donor cell to form a nuclear transfer unit,
  - (b) culturing the nuclear transfer unit until greater than the 2-cell developmental stage; and
  - (c) transferring the cultured nuclear transfer unit to a host non-human mammal of the same species such that the nuclear transfer unit develops into a fetus.
22. A method as claimed in claim 21, wherein the fetus develops into an offspring.
23. A method as claimed in any one of claims 19 to 22, wherein the donor cell nucleus is from mesoderm, endoderm, or ectoderm.
24. A method as claimed in any one of claims 19 to 23 wherein the non-human mammal is bovine, ovine, porcine, equine, caprine and buffalo.
25. A method as claimed in any one of claims 19 to 24, wherein the donor cell nucleus is from

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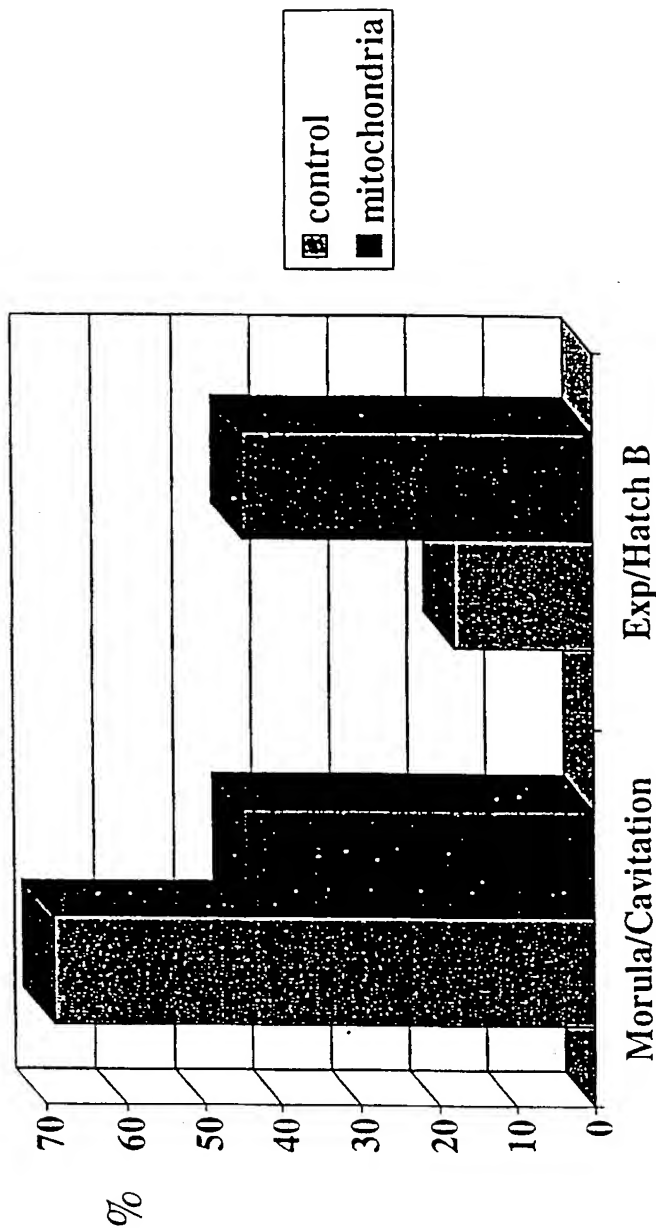
epithelial-cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, fibroblasts, or muscle cells.

- 5 26. A method as claimed in any one of claims 19 to 25, wherein the donor cell nucleus is from an organ selected from the group consisting of skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organ, bladder, kidney and urethra.
27. A method as claimed in any one of claims 19 to 26, wherein the enucleated recipient oocyte is matured in vitro or in vivo prior to enucleation.
- 10 28. A method as claimed in any one of claims 19 to 27 wherein the enucleated recipient oocyte is a Metaphase II stage oocyte.
29. A method as claimed in any one of claims 19 to 28 wherein the donor nucleus is membrane-bounded
30. A method as claimed in any one of claims 19 to 29 wherein the donor nucleus is a whole blastomere.
- 15 31. A method as claimed in any one of claims 19 to 30 wherein the donor nucleus is a karyoplast aspirated from a blastomere.
32. A recipient oocyte comprising a perivitelline space, and a donor cell nucleus and replicative mitochondria deposited in the perivitelline space.
- 20 33. A recipient oocyte as claimed in claim 32 wherein the replicative mitochondria is derived from the same species and cell type as the donor cell nucleus or from the same individual from which the donor cell nucleus is derived.

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# FIGURE 1

Effect of mitochondria injection on  
preimplantation embryo development



Embryos injected at zygote stage (d 0.5) and assessed at day 3.5

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(54) Title: METHODS AND COMPOSITIONS FOR ENHANCING DEVELOPMENTAL POTENTIAL OF OOCYTES AND ZYGOTES

(57) Abstract: The invention relates to compositions and methods for enhancing the developmental potential of oocytes or zygotes by increasing intracellular levels of replicative mitochondria in the oocytes or zygotes. In one aspect of the invention, the intracellular levels of replicative mitochondria are increased by introducing replicative mitochondria into the oocytes or zygotes. The oocytes may be fertilized to obtain a zygote with increased intracellular levels of replicative mitochondria. The methods and compositions may be used to improve in vitro fertilization and embryo transfer methods, and nuclear transfer techniques.

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>VAN BLERKOM JONATHAN ET AL: "Mitochondrial transfer between oocytes: Potential applications of mitochondrial donation and the issue of heteroplasmy." HUMAN REPRODUCTION (OXFORD), vol. 13, no. 10, October 1998 (1998-10), pages 2857-2868, XP001002313 ISSN: 0268-1161 cited in the application the whole document</p> <p style="text-align: center;">--- -/--</p>	1-5,10

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>TAKEDA K ET AL: "Dominant distribution of mitochondrial DNA from recipient oocytes in bovine embryos and offspring after nuclear transfer."</p> <p>JOURNAL OF REPRODUCTION AND FERTILITY, vol. 116, no. 2, June 1999 (1999-06), pages 253-259, XP001002337</p> <p>ISSN: 0022-4251</p> <p>the whole document</p>	1-33
A	<p>MORITA YUTAKA ET AL: "Oocyte apoptosis: Like sand through an hourglass."</p> <p>DEVELOPMENTAL BIOLOGY, vol. 213, no. 1, pages 1-17, XP002168146</p> <p>ISSN: 0012-1606</p> <p>the whole document</p>	1-33
P,X	<p>PEREZ GLORIA I ET AL: "Further studies on the role of mitochondria in controlling oocyte apoptosis."</p> <p>BIOLOGY OF REPRODUCTION, vol. 62, no. Supplement 1, 2000, page 132</p> <p>XP001002323</p> <p>Thirty-Third Annual Meeting of the Society for the Study of Reproduction; Madison, Wisconsin, USA; July 15-18, 2000</p> <p>ISSN: 0006-3363</p> <p>the whole document</p>	1-33

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